Evidence of udder excretion of *Campylobacter jejuni* as the cause of milk-borne campylobacter outbreak

By D. N. HUTCHINSON, F. J. BOLTON, P. M. HINCHLIFFE,
H. C. DAWKINS

Public Health Laboratory, Preston

S. D. HORSLEY, E. G. JESSOP

South Cumbria Health Authority, Barrow-in-Furness,

P. A. ROBERTSHAW

South Lakeland District Council, Ulverston

AND D. E. COUNTER

Veterinary Investigation Centre, Calthwaite, Penrith

SUMMARY

We describe a community outbreak of campylobacter enteritis associated with the consumption of untreated milk, apparently contaminated by two cows with campylobacter mastitis. The outbreak occurred in two phases. Strains of *Campylobacter jejuni* of the Penner serogroup complex 4, 13, 16, 50 and Preston biotype code 6100 were isolated from patients in both episodes and from the faeces of the cattle, milk filters, bulk milk and retail milk. Milk samples from two of 40 milking cows were found to contain *C. jejuni*, and the wheys from these two cows had high titres of antibody to *C. jejuni* detected by ELISA techniques.

INTRODUCTION

Over 27 sizable outbreaks of campylobacter infection associated with the consumption of raw or inadequately treated milk have been reported in the United Kingdom (Robinson & Jones, 1981; Communicable Disease Surveillance Centre, London, unpublished). Elsewhere, milk or milk products have been implicated in nine outbreaks in the U.S.A. (Blaser, Taylor & Feldman, 1983), one in Canada (McNaughton, Leyland & Mueller, 1982) and another in Switzerland which affected over 500 joggers given a drink made with raw milk (Stalder *et al.* 1983). The largest outbreak (in Britain) affected an estimated 3500 people (Jones *et al.* 1981).

It is thought that in most milk-borne outbreaks campylobacters get into milk by contamination with the faeces of campylobacter infected cows. The experimental inoculation of *Campylobacter jejuni* into cows' udders with the subsequent development of a mastitis, and the finding that the organism was excreted in the milk for several days (Lander & Gill, 1980) indicated an alternative method of

contamination of milk. However, evidence of a naturally occurring mastitis has not been reported and is difficult to obtain.

We describe the investigation of a milk-borne campylobacter outbreak which showed that excretion of the organism in the milk was the most probable route by which bulk milk was contaminated. We also discuss the dilemma facing a Medical Officer for Environmental Health who, when confronted with what is apparently a milk-borne campylobacter outbreak, has to decide if and when to apply a treatment order (Regulation 20, The Milk & Dairies General Regulations, 1959) and when to remove it.

METHODS

The outbreak

On Thursday, 9 June 1983 three inhabitants of a small village fell ill with diarrhoea. By the following Monday, 13 June, 30 people had reported to the local medical practitioner – a victim himself – with symptoms of campylobacter infection. Confirmation of the infecting agent was obtained on 16 June, when *C. jejuni* was isolated from the faeces of the first eight patients sampled. Concurrently newly affected cases were reported from neighbouring villages and from amongst a group of children camping several miles from the first village.

The explosive and widespread nature of the outbreak pointed to the water or milk supply as a possible source of infection. The main water supply to the village had a good record of adequate chlorination and a telephone enquiry to the Water Authority confirmed that there had not been a recent treatment failure. Two samples of mains water were examined during the initial phase of the outbreak and found to be negative for coliforms and campylobacters. On the other hand, in the affected villages, virtually all the inhabitants drank unpasteurized milk from a local supplier. Moreover, symptoms developed in all the members of a household who had moved from their village to a small town several miles away, but who had retained their former supplier of untreated milk. Investigations at the dairy farm supplying the milk (described fully below) showed extensive environmental contamination with campylobacters, but samples of milk and milk filters yielded none. Thus, proof that the milk was the vehicle of infection was not obtained.

The outbreak appeared to have ended by 20 June, by which date 51 people were found to be affected (Fig. 1). By the 22 June there was some public opposition to further investigation of the suspect farm. However, between the 25 June and 2 July, 24 more people developed diarrhoea. Milk sampling was intensified and *C. jejuni* was isolated from a milk filter collected on 2 July. A treatment order was applied on the 4 July and enforced until 25 July, when three consecutive samples of milk and milk filters had not grown campylobacters. During this period the villagers obtained raw milk from a different dairy! No new cases of campylobacter enteritis were recognized after 4 July, although a sporadic case of enteritis due to *Salmonella typhimurium* was recorded on 10 July. Follow up of cases continued until 24 August.
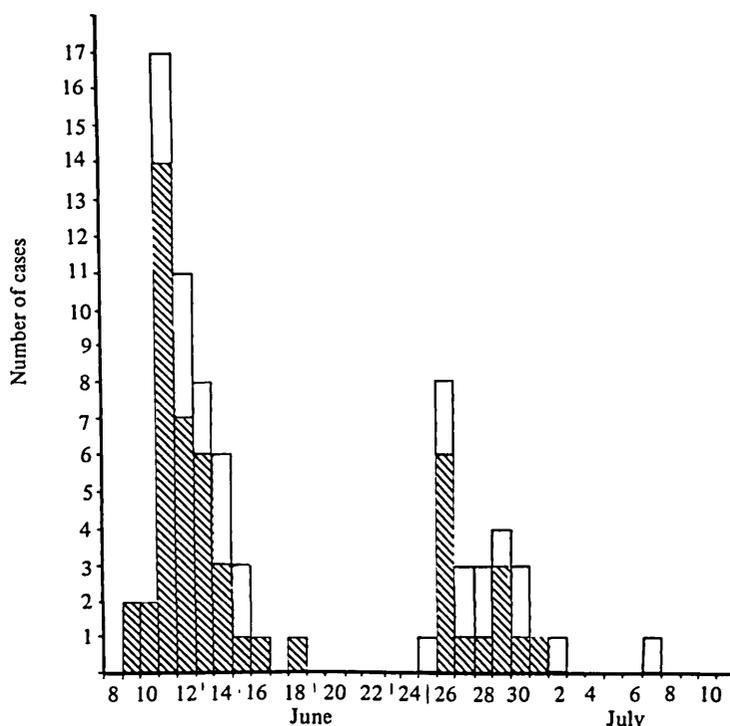
Fig. 1. Date of onset of symptoms in culture-positive (shaded area) and negative cases (*n* = 75).

Bacteriological methods

Culture

Faecal samples. Human faecal samples were cultured for campylobacters directly on plates of Preston agar (Bolton & Robertson, 1982), incubated microaerobically at 42 °C for 48 h. All specimens were examined for the presence of salmonellae and shigellae by routine laboratory methods. Animal faecal samples were cultured for campylobacters in Preston enrichment broth (6 ml) (Bolton *et al.* 1983) which were incubated at 42 °C for 24 h and then subcultured onto Preston agar, incubated as described above.

Milk filters. During the first phase of the outbreak (14–22 June) milk filters were collected in dry sterile containers (without enrichment broth) and sent to the laboratory, where on receipt they were cultured in Preston broth (25–30 ml). In the second phase they were placed directly into the enrichment broth on the farm. Filters collected after evening milkings (in both phases) were kept at 4 °C overnight for delivery the next day.

Bulk and retail milk. Bulk milk samples of about 500 ml and retail milk samples of about 1·5 l (prepared by pooling 250 ml samples from each of 5 or 6 bottles) were filtered through pads of surgical gauze, which were then cultured for campylobacters in 25–30 ml of enrichment broth as described above.

Milk samples from cows. After discarding the foremilk the teats were cleansed with alcohol before collecting about 10 ml of milk aseptically from each quarter into a common container; 25 ml of each cow's sample were added to an equal

volume of single strength enrichment broth which was cultured for campylobacters as before.

Environmental samples. Midden samples were treated as animal faeces. Surface swabs from the dairy and bottling areas were broken off into bottles of enrichment broth. Water samples (1 l) were cultured by the filtration method described by Bolton *et al.* (1982).

Identification, biotyping and serotyping of campylobacter isolates. Campylobacters were identified by typical colonial morphology, positive oxidase test, and motility and cell morphology as shown by dark field microscopy. Isolates were biotyped according to the Preston scheme (Bolton, Holt & Hutchinson, 1984) and serotyped according to the Penner scheme (Penner & Hennessy, 1980) by Dr D. M. Jones of the Public Health Laboratory, Manchester.

Detection of antibody in whey. Milk samples from individual cows were treated with rennet and the whey stored at -20 °C. Control wheys were prepared from bulk milk samples which were brought to the laboratory for routine statutory tests. The titre of anti-campylobacter antibodies was determined by a conventional ELISA technique (Voller, Bidwell & Bartlett, 1979) and also by the COMPELISA technique (Hinchliffe & Robertson, 1983) for the detection of complement fixation. Reagents were used in 100 μl volumes throughout. For both tests round-bottomed 96-well microtitration plates were coated by adsorption overnight at 4 °C with an antigen prepared by sonicating a composite of several *C. jejuni* and *C. coli* strains. The optimum dilution of this extract in carbonate-bicarbonate buffer, pH 9·6, was found to be 1/200. Excess antigen was removed after adsorption by washing with phosphate buffered saline (PBS)/Tween 20 buffer, pH 7·4.

The whey samples were diluted in PBS/Tween 20 buffer to 1/100 for the ELISA and 1/20 for the COMPELISA. After inactivation of the latter serum dilution at 56 °C for 30 min, they were allowed to react with the solid phase antigen for 1 h at room temperature (RT). All tests were done in duplicate.

In the COMPELISA this reaction was followed by the addition of guinea-pig serum as a source of complement. After incubation at RT for 1 h unfixed complement was washed away. Any complement that had been fixed by any antigen-antibody complex was detected by the addition of an anti-guinea-pig C3 antiserum which had been conjugated with peroxidase (Cappell Laboratories).

In the ELISA, immunoglobulin that had reacted with the antigen was detected directly with an anti-bovine IgG antibody conjugated with peroxidase (Miles Laboratories). In bot tests conjugates were allowed to react at RT for 2 h.

Subsequent addition of citric acid-phosphate buffer, pH 5·0 containing hydrogen peroxide as substrate and ortho-phenylenediamine as chromogen resulted in a colour reaction, which was stopped after 15 min incubation at RT by the addition of 50 μl volumes of 2 M-H_2SO_4. The intensity of the colour was measured in a Dynatech MR 58 Micro-ELISA reader.

RESULTS

Investigation of patients and population

The date of onset of symptoms in those reporting ill is shown in the histogram (Fig. 1). This illustrates the bimodal distribution of the outbreak: phase I was from

Table 1. *Association of campylobacter culture and symptoms**

Category	Number of persons
Culture positive	
With symptoms	50
Without symptoms	10
Culture negative	
With symptoms	25
Without symptoms	33
Total	118

* Included diarrhoea, vomiting, raised temperature and abdominal pain.

Table 2. *Symptoms presented by 75 people from culture positive and culture negative groups*

Symptoms	Culture positive ($n = 50$)	Culture positive ($n = 25$)
Diarrhoea	47	19
Vomiting	11	5
Raised temperature	7	3
Abdominal pain	8	6
Headache shivering	1	3

Table 3. *Duration of* C. jejuni *excretion in 61 patients*

Period of excretion (days)*	Number of patients
7	1
7–14	16
14–21	23
21–28	14
28	7
Total	61

* Determined from the time of onset of illness to the first culture-negative stool specimen.

9 June to 18 June and phase II from 25 June to 7 July. Faecal samples and clinical details were obtained from 75 persons reporting symptoms and 56 of their household contacts. In all, 269 faecal samples were examined, of which 131 were acute or initial screening specimens and 138 were convalescent or follow-up specimens. All were negative for salmonellae and shigellae. *C. jejuni* was isolated from 50 (67 %) of the 75 symptomatic patients and 10 of their symptomless contacts. The association of symptoms with the isolation of *C. jejuni* is shown in Table 1. The incidence of the various symptoms in the *C. jejuni* positive and negative patients was not significantly different (Table 2). The duration of *C. jejuni*

excretion in 61 people is shown in Table 3 : 7 (11 %) were still excreting the organism one month after the onset of illness.

During the outbreak 65 unselected households were investigated. In 41 of them there was at least one patient who was culture positive for *C. jejuni*. In the first phase of the outbreak, 25 of 35 housholds were positive and in the second phase 16 of 30 were positive. All of these homes received untreated milk from the same supplier.

All isolates were resistotyped and a representative sample serotyped. Twenty-six of the isolates which were serotyped proved to be *C. jejuni* serotype 50 or the antigenically related 4, 13, 16, 50 complex. The other isolate from a symptomless farm worker was an unrelated serotype 3. Apart from this latter strain all human isolates from both phases of the outbreak gave the biotype code 6100.

Investigation of the dairy farm

The dairy farm supplying the milk was visited on numerous occasions during the outbreak. There had been no recent illness among the 47 head of cattle, of which 40 were in milk, and sheep were the only other stock on the farm. The farm buildings were old, but adequate, clean and well ventilated. Mechanical milking was carried out twice daily with the animals in their over-wintering stalls. The milk was pumped to a separate room where, after cooling, it was stored in churns before being trundled across the farmyard to the dairy which adjoined the farm house. The dairy was a flagstone-floored room measuring about 16 metres square. It housed a bottle washer and semi-automated bottle filling machine – bottles were capped by hand. The bottling and retailing of the milk was done by a man and wife to whom the farmer sold the milk, an arrangement that had been operating for several years. The local Environmental Health Department were not aware of any previous outbreak of illness associated with this farm's milk and routine statutory tests of the milk had always been satisfactory.

Milk filters and milk samples

A total of 40 milk filters from morning and evening milkings were collected for culture throughout the period of the investigation. In addition, 11 bulk milk samples were collected on 5 days during the later part of the outbreak.

Twelve milk filters collected between June 14 and June 22 were all culture negative. However, *C. jejuni* was isolated from milk filters on the 2, 4, 8 and 10 of July, and from bulk milk on the 11 and 13 July. The biotype code and serogroups of these isolates corresponded with those from human faeces.

Two of five pooled retail milk samples collected on Jun 29 and 30 were also *C. jejuni* positive. Both isolates possessed serogroup antigens 13 and 50 expressed to different degrees, and gave the biotype code 6100. Of 40 milk samples collected from individual cows' on the 7 July, 2 were positive for *C. jejuni*. These strains were biotyped (code 6100 and 6000), but the cultures died before serotyping was completed.

Animal faecal samples

C. jejuni was isolated from 4 of 21 sheep faecal samples. These isolates were serotypes 1 and 23 and therefore different to the outbreak strain. Twenty-one of
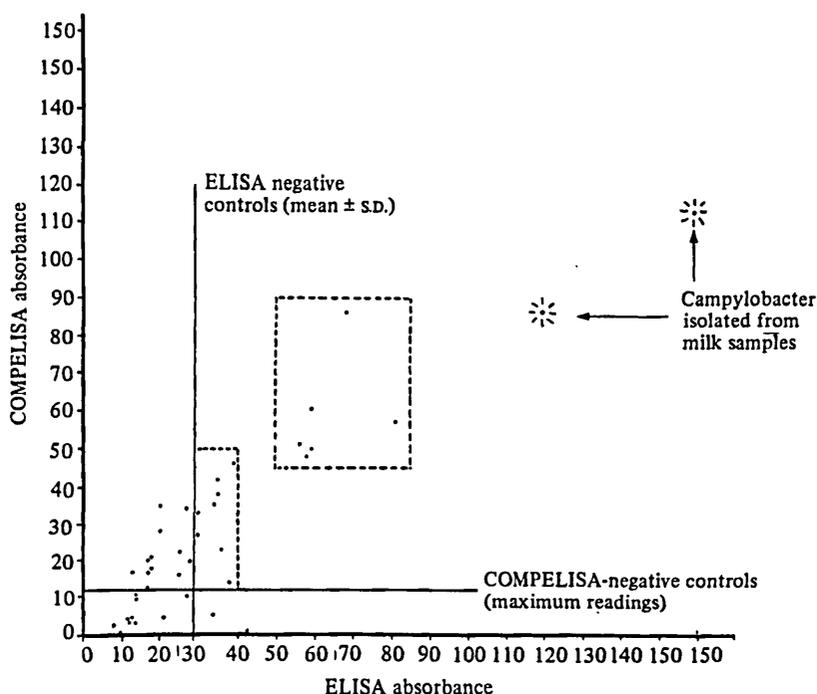
Fig. 2. Whey antibody concentrations detected by ELISA and COMPELISA in milk samples from individual cows of the outbreak herd.

the 47 cattle (40 in milk) and calves on the farm were found to be excreting *C. jejuni* when sampled on July 7. These 21 isolates included serotypes 1, 2, 23, 48 and 50 and an untypable strain. Eighteen of the 40 cows in milk were positive for *C. jejuni*. The predominating serotypes were of the serogroup complex, 4, 13, 16, 50 (excreted by 9 cows) and type 23 (excreted by 6 cows). One cow was excreting two different serotypes of *C. jejuni*.

Environmental samples

These included midden samples, untreated water from field watering holes and tanks or troughs on the farm, chlorinated water from the dairy and bottling areas, surface swabs from the dairy and bottling areas and milk bottle rinses. Midden samples collected on the 18, 22 and 24 June were positive for *C. jejuni*. Several serotypes were isolated, including those of the 4, 13, 16, 50 complex. Two of the four raw watering samples collected from watering holes in the fields were positive for *C. jejuni* (serotypes 6·7 and 23). All other environmental specimens were culture negative for campylobacters.

Whey antibody ELISA test

Whey was prepared from milk samples from 40 cows of the outbreak herd. The absorbance readings of the ELISA and COMPELISA tests were compared with those obtained from 26 control wheys. The mean and standard deviation of control wheys were 16·2±6·5 with a range of 1–30. Twenty of the control wheys in the

COMPELISA had absorbances of < 1; the remaining 6 had absorbances which ranged from 2 to 12. The results of tests on the outbreak herd showed 8 cows with low levels of antibody, 6 cows with moderate levels of antibody, and 2 cows with high levels of antibody (Fig. 2). Milk from the latter 2 cows yielded *C. jejuni* on culture.

DISCUSSION

Most campylobacter milk-borne outbreaks are explosive, but several reports suggest that contamination can be intermittent over a prolonged period and that a mixture of serogroups may be present simultaneously (Jones, Robinson & Eldridge, 1981; Wilson *et al.* 1983). The biphasic pattern of this outbreak indicates contamination of the milk on at least two separate occasions. The human isolates in both episodes of the outbreak belonged to the serogroup complex 4, 13, 16, 50 and biotype code 6100. Despite the minor differences in the expression of the different antigens in the 4, 13, 16, 50 serogroup complex it is our opinion that all human isolates were the same strain. Although several different serogroups and biotype codes were isolated from the farm animals and environment, many isolates from cattle and milk were of the same serogroup and biotype code as the outbreak strain.

C. jejuni strains of Preston biotypes codes 6100 and 6000 were isolated from individual milk samples of two cows. Neither animal had signs of mastitis nor had it been ill recently. When additional individual milk samples were collected from these two animals 10 days later the organism was no longer found. This is not unexpected as experimental *C. jejuni* infection of cows' udders has shown a wide spectrum of illness ranging from acute severe mastitis to mild inapparent disease; moreover, in a high milk yielding cow, excretion of the organisms may be as short as 3 days (Lander & Baskerville, 1983). Contamination of the two milk samples was a slight possibility, as both animals were faecal excretors of *C. jejuni* of the same biotype codes as those isolated from their milk. Had cell excretion counts of the individual milk samples been done, it would have helped to differentiate infection from contamination. However, the likelihood that the isolates were excreted in the milk was supported by the results of the antibody studies on the individual milk samples.

In the whey ELISA test, 8 of the 40 cows had moderate or high levels of antibody. It was only when the results of the isolation and the whey antibody studies, which had been carried out independently, were collated that we discovered that the animals with the highest titres were the cows whose milk contained *C. jejuni*. Drawing a parallel from experience with a whey CFT in brucellosis (Farrell & Robertson, 1968) it is reasonable to conclude that the two animals had recently had *C. jejuni* udder infections and that this could have been the sole source of the contaminated milk in this outbreak.

It is of interest that 21 % of human cases were culture negative. This may reflect the delays in obtaining specimens from some subjects caused by the logistical problems of investigating a large outbreak in a scattered rural area. Hutchinson & Bolton (1983) showed that enrichment culture of faeces increases the number of campylobacer isolations, especially in asymptomatic or convalescent cases and in specimens delayed in transit. We did not use enrichment cultures in this study, but we recommend their use in all future campylobacter outbreaks.

The isolation of campylobacters from milk filters of farms associated with outbreaks is not uncommon (Robinson & Jones, 1981). However, in most investigations – as in this one – an isolation is obtained only after the outbreak has ended and usually after several negative results (Robinson *et al.* 1979). Our failure to isolate *C. jejuni* from filters until after the second episode is attributable to a change of technique. Initially the filters were held in dry sterile containers for several hours pending delivery to the laboratory, but later they were placed in campylobacter enrichment medium at the farm immediately milking had been completed. This would explain why milk filters collected from evening milkings, which were placed in the enrichment broth within 30 min were positive, whereas the morning specimens which were often delayed for 3 h before being placed into broth, were negative. It is possible that failure to dilute out the natural antibacterial effect of fresh milk quickly (Wilson, Miles & Parker, 1983) may have reduced survival of campylobacters in the morning samples.

C. jejuni has seldom been isolated from bulk milk samples associated with outbreaks, but two recent surveys have detected the organisms in 0·9 % (Doyle & Roman, 1982) and 1·5 % (Lovett, Francis & Hunt, 1983) of routinely sampled bulk raw milk. In most studies low success rates probably reflect low levels of contamination through dilution effected by bulking milk. In the small herd studied in this outbreak these effects would have been less pronounced, with the result that we were able to isolate *C. jejuni* from bulk milk sampled over 4 days. As far as we can determine there has not been a previous report of the isolation of *C. jejuni* from retail bottled milk. Two of the five pooled samples from 26 bottles contained *C. jejuni* of the same serogroup as those isolated from human cases. These isolations were made late in the outbreak and consumption of this batch of milk could not be linked with any human infection, even though the treatment order had not been imposed. Belatedly we attempted to enumerate the campylobacters in these samples using the most probably number (MPN) technique (Bolton *et al.* 1982) but unfortunately the samples gave negative results, probably because of prolonged storage.

In Britain, although a treatment order can be served without having bacteriological evidence of contamination of the milk, there is an understandable reluctance by many Medical Officers of Environmental Health to do so, particularly as its imposition is damaging to the farmer and unwelcome to the public, despite strong circumstantial evidence implicating milk as the vehicle. After the second wave of cases the decision to apply the order had been taken before campylobacters had been isolated from the milk; it was coincidental that a positive isolation was made the same day as the treatment order was enforced. Having served an order, what criteria should be applied to allow its removal? In this outbreak the order was lifted when campylobacters were not isolated from three consecutive daily samples of bulk milk and milk filters. These measures may not be sufficient because detectable contamination may be intermittent. In this episode and an outbreak in Bradford (Robinson *et al.* 1979) *C. jejuni* was isolated from milk filters 34 and 26 days respectively after the onset of the outbreak. The dilemma that may face those responsible for public health will be resolved only by making the sale of raw milk illegal. Such legislation was passed in Scotland in 1983, but in the rest of Britain it is long overdue.

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